Table of Contents

1. Overview of GeneBLAzer® Technology

GeneBLAzer® Beta-lactamase Reporter Technology provides a highly accurate, sensitive, and easy to use method of monitoring cellular responses to drug candidates or other stimuli (1). The core of the GeneBLAzer® Technology is a Fluorescence Resonance Energy Transfer (FRET) substrate that generates a ratiometric reporter response with minimal experimental noise. In addition to the two-color (blue/green) readout of stimulated and unstimulated cells, this ratiometric method reduces the absolute and relative errors that can mask the underlying biological response of interest. Such errors include variations in cell number, transfection efficiency, substrate concentration, excitation path length, fluorescence detectors, and volume changes. The GeneBLAzer® Beta-lactamase Reporter Technology has been proven effective in high-throughput screening (HTS) campaigns for a range of target classes, including G-protein coupled receptors (GPCRs) (2, 3), nuclear receptors (4-6), and kinase signaling pathways (7).

2. Materials Supplied

Cell Line Name:	SIE-*bla* HEK 293T
Description:	This cell line utilizes GeneBLAzer® beta-lactamase technology. CellSensor™ SIE-bla HEK 293T cells contain a beta-lactamase reporter gene under control of the SIE-Inducible Element (SIE), stably integrated into HEK 293T cells. This cell line can be used to detect agonists/antagonists of the STAT 3 signaling pathway. SIE-bla HEK 293T cells have been shown to respond to Interleukin-6 (IL-6).
Product Number:	K1649
Shipping Condition:	Dry Ice
Storage Condition:	Liquid Nitrogen ***Note:*** Immediately on receipt, cells must be stored in liquid nitrogen. Cells can be safely thawed 24 hours after placement in liquid nitrogen. Cells **cannot** be stored at –80°C, as they will quickly lose viability.
Quantity:	Refer to Certificate of Analysis (CofA)
Application:	This cell line can be used to detect agonists/antagonists of the STAT 3 signaling pathway.
Growth Properties:	Adherent, doubling time 25–35 hours
Propagation	Typically feed or split 1:4–1:15 2–3 times per week. Cells may appear clumpy in culture and during cell counts. Gently pipet up and down to disperse clumps.
Cell Phenotype:	Epithelial
Selection Marker:	Blasticidin (5 µg/mL)
Vector Used:	pLenti-*bsd*/ SIE-*bla* Vector
Response Element Sequence:	5X GGTTCCCGTAAATGCATCA
***Mycoplasma* Testing:**	Negative
Biosafety Level:	2

3. Materials Required, but Not Supplied

Media/Reagents	Recommended Source	Part Number*
LiveBLAzer™ –FRET B/G Loading Kit, containing: LiveBLAzer™ -FRET B/G Substrate (CCF4-AM), DMSO, Solution B, and Solution C	Invitrogen	K1095 (0.2 mg) K1096 (1 mg) K1030 (5 mg)
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
DMEM (high-glucose) with GlutaMAX™	Invitrogen	10569-010
DMSO	Fluka	41647
Fetal bovine serum (FBS), dialyzed, tissue-culture grade (**DO NOT SUBSTITUTE!**)**	Invitrogen	26400-044
Non-essential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
Phosphate-buffered saline without calcium and magnesium [PBS (-)]	Invitrogen	14190-136
HEPES (1 M, pH 7.3)	Invitrogen	15630-080
Human Interleukin-6 (IL-6)	R&D Systems	206-IL
0.05% Trypsin/EDTA	Invitrogen	25300-054
Blasticidin (antibiotic)	Invitrogen	R210-01

Consumables	Recommended Source	Part #
Black-wall, clear-bottom, 384-well assay plates (with low fluorescence background)	Corning Life Sciences	3712
Compressed air	Various	—

Equipment	Recommended Source
Fluorescence plate reader with bottom-read capability	Various
Filters, if required for plate reader (see **Section 5.3**)	Chroma Technology Corp.
Optional: Epifluorescence- or fluorescence-equipped microscope with appropriate filters	Various
Optional: Microplate centrifuge	Various

Note: For step-by-step instrument setup guides, visit www.invitrogendrugdiscovery/instrumentsetup.

Note: If you do not have access to a fluorescence plate reader with bottom-read capabilities, contact our Technical Support at 1-760-603-7200, select option 3 and enter extension 40266. The assay conditions of this cell line have been fully validated with LiveBLAzer™ FRET B/G substrate and bottom-reading instruments. Other beta-lactamase substrates and top-reading method have not been tested with this cell line.

* Some part numbers differ outside of the continental U.S. Please check with your local Invitrogen Technical Support.

** The clone used to develop this cell line was selected from growth conditions using of dialyzed fetal bovine serum (FBS). Other forms of FBS may kill the cells, especially upon first thaw.

4. Cell Culture Conditions

4.1 Media Required

Component	Thaw Medium	Growth Medium	Assay Medium	Freezing Medium
DMEM (high glucose)	500 mL bottle	500 mL bottle	500 mL bottle	—
FBS, dialyzed	50 mL	50 mL	50 mL	—
HEPES (1 M)	12.5 mL	12.5 mL	12.5 mL	—
NEAA (10 mM)	5 mL	5 mL	5 mL	—
Penicillin (10,000 U/mL) / Streptomycin (10,000 µg/mL)	5 mL	5 mL	5 mL	—
Blasticidin	—	5 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Note: Unless otherwise stated, warm all media and solutions to at least room temperature before adding them to the cells (we recommend 37°C for optimal performance).

Note: We prepare our media by adding the listed components directly to the medium bottle. Alternatively, blasticidin can be added directly to the cell culture flask to reach 5 µg/mL. Similar methods may be suitable.

4.2 Growth Conditions

4.2.1 Special Considerations for Working with this Cell Line

1. This cell line is a clonal population isolated by Fluorescence Activated Cell Sorting (FACS) based on the functional response to Interleukin-6 (IL-6). Assay performance can be expected to depend upon use of the specified media, as responsive cells have been chosen based on these formulations.
2. Cells should double every 25–35 hours.
3. Typically feed or pass cells 1:4–1:15 at least twice a week, and maintain them in a 37°C/5% CO_2 incubator.
4. Cells may appear clumpy in culture and during cell counts. Gently pipet up and down to disperse clumps.
5. This cell line is blasticidin resistant.
6. For additional information about the HEK293T cellular background, please contact Technical Support at the number at the bottom of this page.
7. Cells should be harvested from the flask at a density of 120,000–200,000 cells per cm^2 on the day of the assay plating.

4.2.2 Thawing Method

1. Place Thaw Medium (without Blasticidin) into a flask (10 mL for a T25 flask, 20 mL for a T75 flask, and 30–40 mL for a T225 flask).
2. Place the flask in a humidified 37°C/5% CO_2 incubator for 15 minutes to allow medium to equilibrate to the proper pH and temperature.
3. Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1–2 minutes. Do not submerge vial in water.
4. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
5. Transfer the vial contents drop-wise into 10 mL of Thaw Medium in a sterile 15-mL conical tube.
6. Centrifuge the cells at 400 × *g* for 5 minutes.
7. Aspirate the supernatant and resuspend the cell pellet in 1 mL of fresh **Thaw Medium**.
8. Count the cells

9. Seed cells at a density of ~40,000–80,000 cells/cm^2 into the flask(s) containing pre-equilibrated **Thaw Medium**. Place flask(s) in a humidified 37°C/5% CO_2 incubator.

10. Switch to **Growth Medium** with Blasticidin once the cells are dividing normally every 25–35 hours.

4.2.3 Propagation Method

1. Cells should be passaged (typically 1:4–1:15) or fed at least twice a week. Cells should be maintained between 10% and 90% confluence. Do not allow cells to reach confluence.

2. To passage cells, aspirate medium, rinse once in PBS, add Trypsin/EDTA (3 mL for a T75 flask, 5 mL for a T175 flask, and 8 mL for a T225 flask) and swirl to coat the cells evenly. Cells usually detach after ~2–5 minutes exposure to Trypsin/EDTA. Add an equal volume of **Growth Medium** to inactivate Trypsin.

3. Verify under a microscope that cells have detached and clumps have completely dispersed. If clumps persist, gently pipet up and down to disperse or spin down and resuspend in **Growth Medium**.

4. Transfer cells to a flask containing fresh **Growth Medium**.

4.2.4 Freezing Method

1. Harvest and count the cells (optimal harvest density of 120,000–200,000 cells/cm^2), then spin cells down and resuspend in 4°C of Recovery™ Cell Culture Freezing Medium at 2×10^6 to 1.6×10^7 cells/mL.

2. Dispense 1.0 mL aliquots into cryogenic vials.

3. Place in an insulated container for slow cooling and store overnight at –80°C.

4. Transfer to liquid nitrogen the next day for storage.

5. Assay Procedure

The following instructions outline the recommended procedure for determining activity of compounds as modulators (agonists or antagonists) of the STAT 3 signaling pathway using beta-lactamase as the readout.

- We recommend using 384-well, black-wall, clear-bottom assay plates with low fluorescence background. We have used Corning Life Sciences 3712.
- We recommend including cell-free control wells on the same plate as test wells for background subtraction. See Section 6, Data Analysis.
- Some solvents may affect assay performance. Assess the effect of a test compound solvent before screening. This cell line has been qualified for DMSO tolerance up to 1%. See validation packet at www.invitrogen.com/cellsensor for the assay performance of this cell line in the presence of various DMSO concentrations. The cell stimulation described below is carried out in the presence of 0.5% DMSO to simulate the effect that a test compound solvent might have on the assay. If you use other solvents and/or solvent concentrations, change the following assay conditions and optimize appropriately.
- Cells should be harvested from the flask at a density of 120,000–200,000 cells per cm^2 on the day of the assay plating.

5.1 Quick Reference Guide

For more detailed protocol information, see **Section 5.2**.

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each condition: Unstimulated, Stimulated, and Cell-free.

Note: Some solvents may affect assay performance. Assess the effect of a test compound solvent before screening. The cell stimulation described below is carried out in the presence of 0.5% DMSO to simulate the effect that a test compound solvent might have on the assay. If you use other solvents and/or solvent concentrations, change the following assay conditions and optimize appropriately.

Agonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Cell-Free Wells	Test Compound Wells
Step 1 Harvest and Plate cells	32 µL cells suspended in Assay Medium (20,000 cells/well)	32 µL cells suspended in Assay Medium (20,000 cells/well)	32 µL Assay Medium (no cells)	32 µL cells suspended in Assay Medium (20,000 cells/well)
Step 2 Incubate Cells	No incubation. Proceed immediately to Step 3.			
Step 3 Add agonist or DMSO	8 µL Assay Medium with 2.5% DMSO	8 µL 5X IL-6 in Assay Medium with 2.5% DMSO	8 µL Assay Medium with 2.5% DMSO	8 µL 5X Test Compounds in 2.5% DMSO
Step 4 Incubate cells with Test Compounds	Incubate in a humidified 37°C/5% CO_2 incubator for 5 hours.			
Step 5 Prepare 6X Substrate Mixture	Combine 6 µL of 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM) + 60 µL Solution B and mix. Add 934 µL Solution C and mix.			
Step 6 Load Substrate Mixture	8 µL per well			
Step 7 Incubate Substrate + cells	2 hours at room temperature in the dark.			
Step 8 Detect Activity	See **Section 5.3**			
Step 9 Analyze data	See **Section 6**			

Antagonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Cell-Free Wells	Test Compound or Control Antagonist* Wells
Step 1 Harvest and Plate cells	32 µL cells suspended in Assay Medium (20,000 cells/well)	32 µL cells suspended in Assay Medium (20,000 cells/well)	32 µL Assay Medium (no cells)	32 µL cells suspended in Assay Medium 20,000 cells/well)
Step 2 Incubate Cells	No incubation. Proceed immediately to Step 3.			
Step 3 Add Antagonist or Test Compounds	4 µL Assay Medium with 2.5% DMSO	4 µL Assay Medium with 2.5% DMSO	4 µL Assay Medium with 2.5% DMSO	4 µL Test Compound or Control Antagonist in Assay Medium with 2.5% DMSO
Optional Step	Incubate plate with Antagonist for 30 minutes.			
Step 4 Add agonist	4 µL Assay Medium with 2.5% DMSO	4 µL 10X IL-6 in Assay Medium with 2.5% DMSO	4 µL Assay Medium with 2.5% DMSO	4 µL 10X IL-6 in Assay Medium with 2.5% DMSO
Step 5 Incubate cells with Test Compounds	Incubate in a humidified 37°C/5% CO_2 incubator for 5 hours.			
Step 6 Prepare 6X Substrate Mixture	Combine 6 µL of 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM) + 60 µL Solution B and mix. Add 934 µL Solution C and mix.			
Step 7 Load Substrate Mixture	8 µL per well			
Step 8 Incubate Substrate + cells	2 hours at room temperature in the dark.			
Step 9 Detect Activity	See **Section 5.3**			
Step 10 Analyze data	See **Section 6**			

5.2 Detailed Assay Protocol

Plate layouts and experimental outlines will vary; in screening mode, we recommend using at least three wells for each control: Unstimulated Control, Stimulated Control, and Cell-free Control.

5.2.1 Precautions

- Work on a dust-free, clean surface. Always handle the 384-well, black-wall, clear-bottom assay plate by the sides; do not touch the clear bottom of the assay plate.
- If pipetting manually, it may be necessary to centrifuge the plate briefly at room temperature (for 1 min. at 14 × *g*) after additions to ensure all the assay components are on the bottom of the wells.
- Perform assay only if cells are dividing normally, every 25–5 hours.

5.2.2 Plating Cells

1. Harvest cells at 60–90% confluency from culture in Growth Medium as described in **Section 4.2.3,** Step 2.
2. Spin down cells at 400 × g for 5 minutes and resuspend in **Assay Medium** at a density of 6.25×10^5 cells/mL.
3. Add 32 µL per well of Assay Medium to the cell-free control wells.
4. Add 32 µL per well of the cell suspension (20,000 cells per well) to Unstimulated and Stimulated wells.
5. No incubation. Proceed to **Section 5.2.3** for an Agonist Assay and **Section 5.2.4** for an Antagonist Assay.

5.2.3 Agonist Assay Plate Setup

Note: This subsection provides directions for performing an agonist assay. Directions for performing an antagonist assay can be found in **Section 5.2.4**.

1. Prepare a stock solution of 2.5% DMSO in Assay Medium.
2. Prepare a 5X Test Compound in Assay Medium with 2.5% DMSO (or if test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 2.5%).
3. Prepare a 5X stock of IL-6 in Assay Medium containing 2.5% DMSO. We recommend running a dose response curve to determine the EC_{100} for your IL-6 solution. See **Section 6.3** for a representative curve.
4. Add 8 µL of the stock solution of 2.5% DMSO in Assay Medium to the Unstimulated Control and Cell-free Control wells.
5. Add 8 µL of the 5X stock of Test Compounds to the Test Compound wells.
6. Add 8 µL of the 5X stock solution of IL-6 to the Stimulated Control wells.
7. Incubate the Agonist assay plate in a humidified 37°C/5% CO_2 incubator for 5 hours. Then proceed to **Section 5.2.5** for substrate loading and incubation.

5.2.4 Antagonist Assay Plate Setup

Note: This subsection provides directions for performing an antagonist assay. Directions for performing an agonist assay are provided in **Section 5.2.3**.

1. Prepare a stock solution of 2.5% DMSO in Assay Medium.
2. Prepare a 10X stock of Test Compounds in Assay Medium with 2.5% DMSO. (Or if test compound is dissolved in DMSO, prepare a 10X stock of Test Compounds in Assay Medium and make sure the DMSO concentration for the 10X solution is 2.5%).
3. Prepare a 10X stock of IL-6 in Assay Medium at an EC_{80} concentration. We recommend running a dose response curve to determine the EC_{80} for your IL-6 solution. See **Section 6.3** for a representative curve.
4. Add 4 µL of the 10X stock of Test Compounds to the Test Compound wells.
5. Add 4 µL of the stock solution of 2.5% DMSO in Assay Medium to the Stimulated Control wells, the Unstimulated Control wells, and the Cell-free Control wells.
6. Add 4 µL of the 10X stock of Control Inhibitor to the Antagonist Control wells.
7. If desired, incubate the Test Compounds with the cells in a humidified 37°C/5% CO_2 incubator before proceeding. Typically, a 30-minute incubation is sufficient.
8. Add 4 µL of the 10X EC_{80} stock solution of IL-6 prepared in Step 3 to the Test Compound wells, the Stimulated Control wells, and the Antagonist Control wells.
9. Add 4 µL of Assay Medium with 2.5% DMSO to the Unstimulated Control and Cell-free Control wells.
10. Incubate the Antagonist assay plate in a humidified 37°C/5% CO_2 incubator for 5 hours. Then proceed to **Section 5.2.5** for Substrate Loading and Incubation.

5.2.5 Substrate Loading and Incubation

Note: This protocol is designed for loading cells with LiveBLAzer™-FRET B/G Substrate (CCF4-AM). If alternative substrates are used, please follow the loading protocol provided with the substrate.

Note: Preparation of 6X LiveBLAzer™-FRET B/G Substrate (CCF4-AM) mixture and cell loading should be done quickly in the absence of direct strong lighting.

1. Prepare Solution A: 1 mM LiveBLAzer™-FRET B/G Substrate (CCF4-AM, MW = 1096) stock solution. Add anhydrous DMSO provided directly to the vial of lyophilized CCF4-AM, using 182 µL of DMSO for every 200 µg of CCF4-AM. Mix well. Store the aliquots of the stock solution at –20°C until use.
2. Prepare 6X LiveBLAzer™-FRET B/G (CCF4-AM) Substrate Mixture:
   - 2.1 Add 6 µL of Solution A to 60 µL of Solution B and vortex.
   - 2.2 Add 934 µL Solution C to the combined solutions from above step with vortexing.
   - *Note:* If more than 1 mL 6X Substrate Mixture is needed, scale up the amount of each solution proportionally.

3. Remove assay plate from the humidified 37°C/5% CO_2 incubator.
4. Add 8 µL of 6X Substrate Mixture from **Step 2** to each well.
5. Cover the plate to protect it from light and evaporation.
6. Incubate at room temperature for 2 hours.

5.3 Detection

Note: All measurements are made at room temperature from the bottom of the wells, preferably in 384-well, black-wall, clear-bottom assay plates with low fluorescence background. Before reading the plate, remove dust from the bottom with compressed air.

Note: For instrument specific setup guides visit our website at www.invitrogen.com/instrumentsetup or contact our Drug Discovery Technical Support at 1-800-955-6288 select option 3 and enter 40266.

5.3.1 Instrumentation, Filters, and Plates

1. Fluorescence plate reader with bottom reading capabilities.
2. Recommended filters for fluorescence plate reader:

   Excitation filter: 409/20 nm
   Emission filter: 460/40 nm
   Emission filter: 530/30 nm
3. Recommended dichroic mirrors: 380 nm, 400 nm, and 425 nm cutoff mirrors have been successfully used, and general 50/50 mirrors may also be suitable.
4. Refer to ToxBLAzer™ protocol for specific instrumentation and filter recommendations.

5.3.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before making measurements.

6. Data Analysis

6.1 Background Subtraction

We recommend that you subtract the background for both emission channels (460 nm and 530 nm).

1. Use the assay plate layout to identify the location of the Cell-Free wells. These control wells are used for background subtraction.
2. Determine the average emission from the Cell-Free wells at both 460 nm (Average Blue Background) and 530 nm (Average Green Background).
3. Subtract the Average Blue Background (data collected at 460 nm) from all of the blue emission data.

Note: Background corrected values should not be near zero.

4. Subtract the Average Green background (data collected at 530 nm) from all of the green emission data.

Note: Background corrected values should not be near zero.

5. Calculate the Blue/Green Emission Ratio for each well, by dividing the background-subtracted blue emission values by the background-subtracted green emission values.

6.2 Visual Observation of Intracellular Beta-lactamase Activity Using LiveBLAzer™-FRET B/G Substrate (CCF4-AM)

***Note*:** Microscopic visualization of cells will cause photobleaching. Always read the assay plate in the fluorescence plate reader before performing microscopic visualization.

An inverted microscope equipped for epifluorescence and either a xenon or mercury excitation lamp is typically required to view the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) signal in cells. To visually inspect the cells, you will need a long-pass filter passing blue and green fluorescence light so that your eye can visually identify whether the cells are fluorescing green or blue.

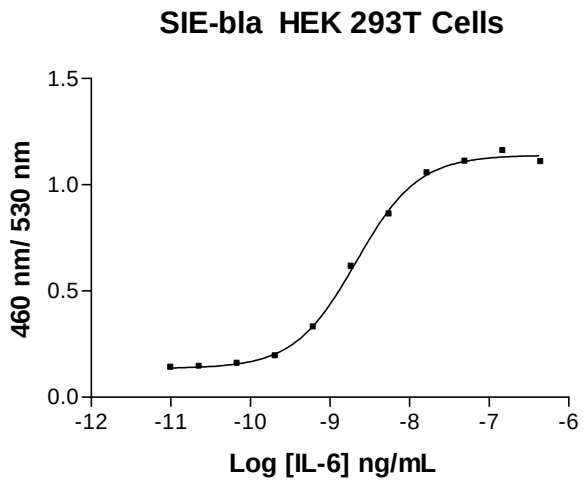
Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (www.chroma.com).

Chroma Set # 41031

Excitation filter:	HQ405/20x (405 ± 10 nm)
Dichroic mirror:	425 DCXR
Emission filter:	HQ435LP (435 long-pass)

Filter sizes vary for specific microscopes and need to be specified when the filters are ordered. For epifluorescence microscopes, a long-pass dichroic mirror is needed to separate excitation and emission light and should be matched to the excitation filter (to maximally block the excitation light around 405 nm, yet allow good transmission of the emitted light).

6.3 Representative Data



EC_{50}	2.1 ng/mL
EC_{80}	7.3 ng/mL
EC_{100}	450 ng/mL
Z′ at EC_{100}	0.81
Assay Window at EC_{100}	7.8

Dose response of SIE-*bla* HEK 293T cells to IL-6. SIE-*bla* HEK 293T cells were plated into a 384-well assay plate at 20,000 cells per well.. Then, the cells were treated with IL-6 over the indicated concentration range in a 384-well format for 5 hours at 37ºC/5% CO_2 in the presence of 0.5% DMSO. Following stimulation, cells were loaded with LiveBLAzer™-FRET B/G Substrate (CCF4-AM) for 2 hours at room temperature. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted against the concentration of the agonist.

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8. Purchaser Notification

Limited Use Label License No. 28: CMV Promoter

The use of the CMV promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned and licensed by the University of Iowa Research Foundation and is sold for research use only. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation (UIRF), 214 Technology Innovation Center, Iowa City, Iowa 52242. For further information, please contact the Associate Director of UIRF, at 319-335-4546.

Limited Use Label License No. 150: GeneBLAzer® Technology

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Use of Genetically Modified Organisms (GMO)

Information for European Customers. The SIE-*bla* HEK 293T cell line(s) are genetically modified with the pLenti-*bsd*/SIE-*bla* Vector. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.